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Inhibition of adipocyte differentiation by RORα

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ABSTRACT

Here we show that gene expression of the nuclear receptor RORα is induced during adipogenesis, with RORα4 being the most abundantly expressed isoform in human and murine adipose tissue. Over-expression of RORα4 in 3T3-L1 cells impairs adipogenesis as shown by the decreased expression of adipogenic markers and lipid accumulation, accompanied by decreased free fatty acid and glucose uptake. By contrast, mouse embryonic fibroblasts from *staggerer* mice, which carry a mutation in the RORα gene, differentiate more efficiently into mature adipocytes compared to wild-type cells, a phenotype which is reversed by ectopic RORα4 restoration.

1. Introduction

White adipose tissue is a central organ for whole-body energy homeostasis. It serves as a buffering depot for excess energy and releases fatty acids upon energy demand [1]. Adipose tissue is also an endocrine organ which secretes numerous adipokines such as leptin and adiponectin, and cytokines such as TNFα, which regulate (patho)physiological processes such as insulin sensitivity, inflammation or food intake. Several transcription factors act in concert to orchestrate the differentiation of pre-adipocytes into adipocytes [2]. Peroxisome Proliferator-Activated Receptor γ (PPARγ) and CCAAT/enhancer-binding proteins (CEBP) α, β and δ are master regulators, which trigger a cascade of transcriptional events hence initiating differentiation and instoring the mature phenotype. Recent evidence however indicates that this process can be modulated positively and negatively by other pathways [3]. For example, we and others have shown that Rev-erbα, a nuclear receptor regulating lipid and bile acid metabolism, the inflammatory process and a core clock machinery gene [4], plays also a modulatory role in the regulation of adipogenesis [5,6]. Indeed, Rev-erbα gene expression is induced during the adipogenic process [5,7] and ectopic Rev-erbα expression in 3T3L1 pre-adipocytes promotes their differentiation into mature adipocytes and enhances lipid storage [5].

The RAR-related orphan receptors (RORs) are nuclear receptors closely related to the Rev-erbs. Rev-erbα and RORα are co-expressed in many tissues. In general, they compete for binding to specific monomeric response elements (RevRE/RORE) hence acting as a ying/yang loop to regulate common target genes in an opposite manner (RORα acts as an activator, whereas Rev-erbα as a repressor) [8–10]. For instance, RORα regulates the expression of several Rev-erbα target genes, thereby playing a role in circadian rhythm maintenance and plasma lipid control [11–17]. RORα also plays a role in muscle lipid metabolism [18], and controls the expression of the gluconeogenic glucose-6-phosphatase gene [19].

Here, we have investigated the role of RORα in adipogenesis and report that RORα is a potent inhibitor of adipocyte differentiation.

2. Materials and methods

2.1. Animals

Inguinal and epididymal fat depots were from wild-type C57BL/6 mice. See Supplementary data for details.

Animal care and experimental procedures were performed according to approved institutional guidelines.

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2.2. Human biopsies

Human fat tissue samples were obtained from healthy adult donors, in agreement with French regulations. All subjects gave written informed consent prior sample collection.

2.3. Viral production and infection

To generate cell lines that constitutively over-express RORα4, the coding sequence of human RORα4 was inserted in the MFG retrovirus plasmid [20] to generate the pMFG-RORα4 plasmid. A similar construct lacking the RORα4 sequence (pMFG-Neo) was used throughout the study as a negative control. Phoenix cells were transfected with these constructs and selected for resistance using the geneticin analog G-418. 3T3-L1 cells were infected with the supernatant from MFG-Neo or -RORα4 infected cells, and G-418-resistant infected cells were used within three passages after infection. See Supplementary data for details.

2.4. Cell culture and differentiation

3T3-L1 cells (ATCC) and MFG-Neo or -RORα4 infected 3T3-L1 cells were cultured and differentiated as previously described [21]. See Supplementary data for details.

2.5. MEF isolation and culture

Mouse embryonic fibroblasts (MEFs) were derived from 13.5-day wild-type and *staggerer* mouse embryos. Adipocyte differentiation was initiated after 2 days confluence. MEFs were differentiated with AmnioMAX-C100 medium (Invitrogen), 7.5% AmnioMAX-C100 supplement, 7.5% fetal calf serum, 0.5 mM IBMX, 1 μM dexamethasone, 5 μM insulin. From day 3 to day 8, cells were incubated with the same AmnioMAXC100 medium with 5 μM insulin. At days 0 and 8, cells were lysed and homogenized for RNA isolation or fixed in 4% paraformaldehyde and stained with Oil-red-O.

2.6. FFA uptake assay

Free fatty acid (FFA) uptake was measured using the commercially available *QBT Fatty Acid Uptake Assay Kit* (Molecular Devices, USA) as per the manufacturer's instructions. See Supplementary data for more details.

2.7. Glucose uptake

Assays were performed as described previously [21]. See Supplementary data for details.

2.8. Gene expression

Total RNA was extracted from tissues or cells and retro-transcribed as described earlier [21]. Gene expression was quantified by quantitative RT-PCR on a Mx4000 apparatus (Stratagene, La Jolla, CA) using specific oligonucleotides as described in the Supplementary data. mRNA levels were corrected to an internal control (cyclophilin) and expressed as mean ± S.D.

2.9. Western blotting

See Supplementary data.

2.10. Statistics

All values are presented as mean ± S.D. Comparison between two groups was performed by student's *t*-test. Comparisons among

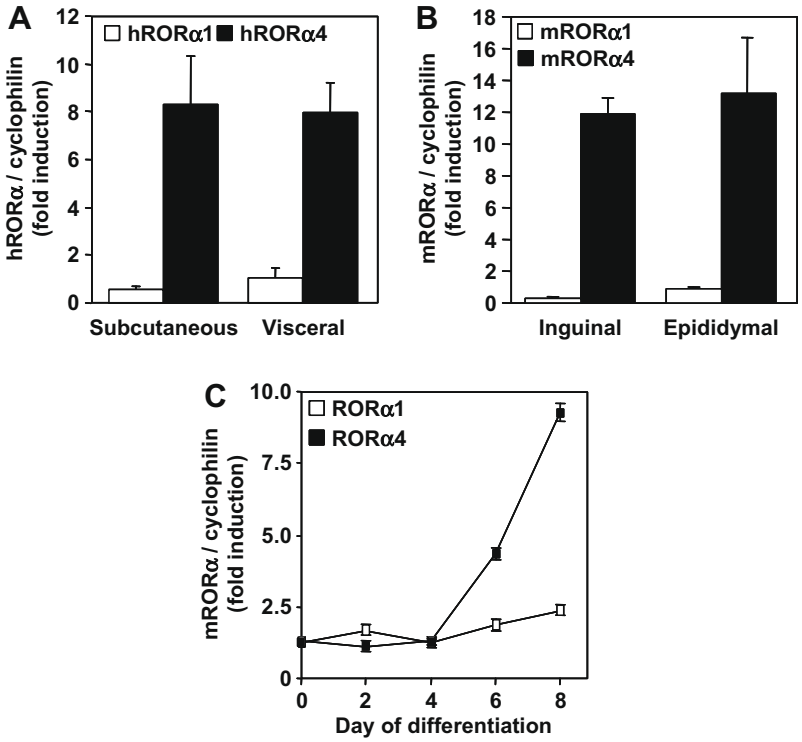

Fig. 1. RORα1 and RORα4 are expressed in adipose tissue and differentiating adipocytes. RORα1 and RORα4 expression in human (A) and mouse (B) adipose tissues, and in differentiating 3T3-L1 cells. (C) mRNA levels were corrected to an internal control (cyclophilin) and expressed as mean ± S.D. of two independent experiments each in triplicate. (A) and (B) RORα1 expression in human visceral and mouse epididymal adipose tissue was set as 1; (C) expression levels of RORα1 and RORα4 were expressed as fold induction of their respective expression level at day 0 set as 1 (absolute Ct values in Table S1).

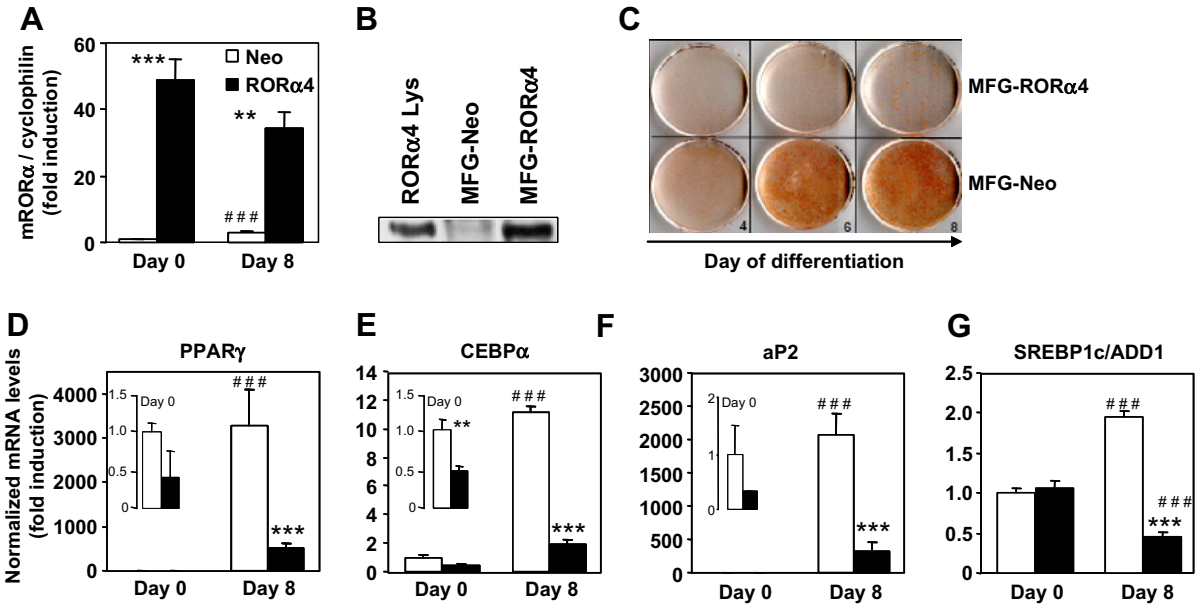

Fig. 2. RORα4 over-expression impairs differentiation of 3T3-L1 pre-adipocytes. RORα4 mRNA (A) and protein (B) levels in 3T3-L1 over-expressing RORα4 (MFG-RORα4) vs control cells (MFG-Neo). Oil-red-O staining (C) and mRNA levels of PPARγ, CEBPα, aP2 and SREBP1c/ADD1 (D–G) upon differentiation. *Difference between Neo and RORα4 over-expressing cells; #difference between day 0 and day 8. Gene expression levels were normalized to an internal control (cyclophilin) and expressed as mean ± S.D. They are from two independent experiments, each realized in triplicate, and expressed as fold induction of the MFG-Neo cells at day 0, set as 1.

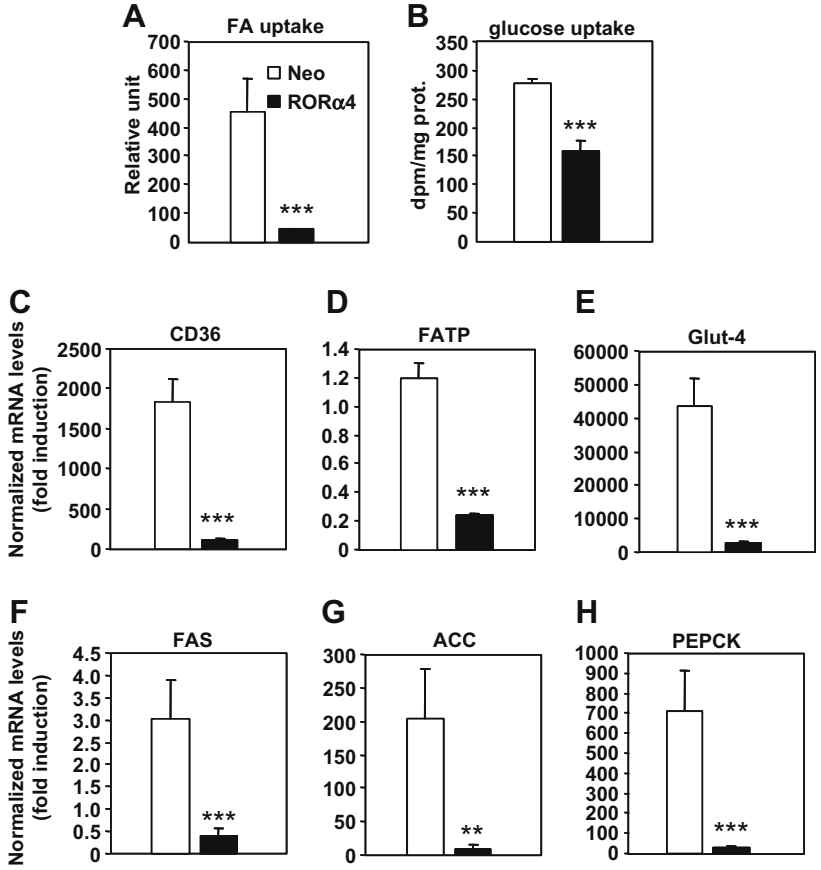

Fig. 3. RORα4 suppresses glucose and free fatty acid uptake in adipocytes. Fatty acid (A) and glucose uptake (B), as well as mRNA levels of genes involved in these processes (C–H) were measured in control and RORα4 overexpressing differentiated 3T3-L1 cells. Uptake data are from three independent experiments realized in triplicate. Gene expression levels (mean ± S.D.) were expressed as in Fig. 2.

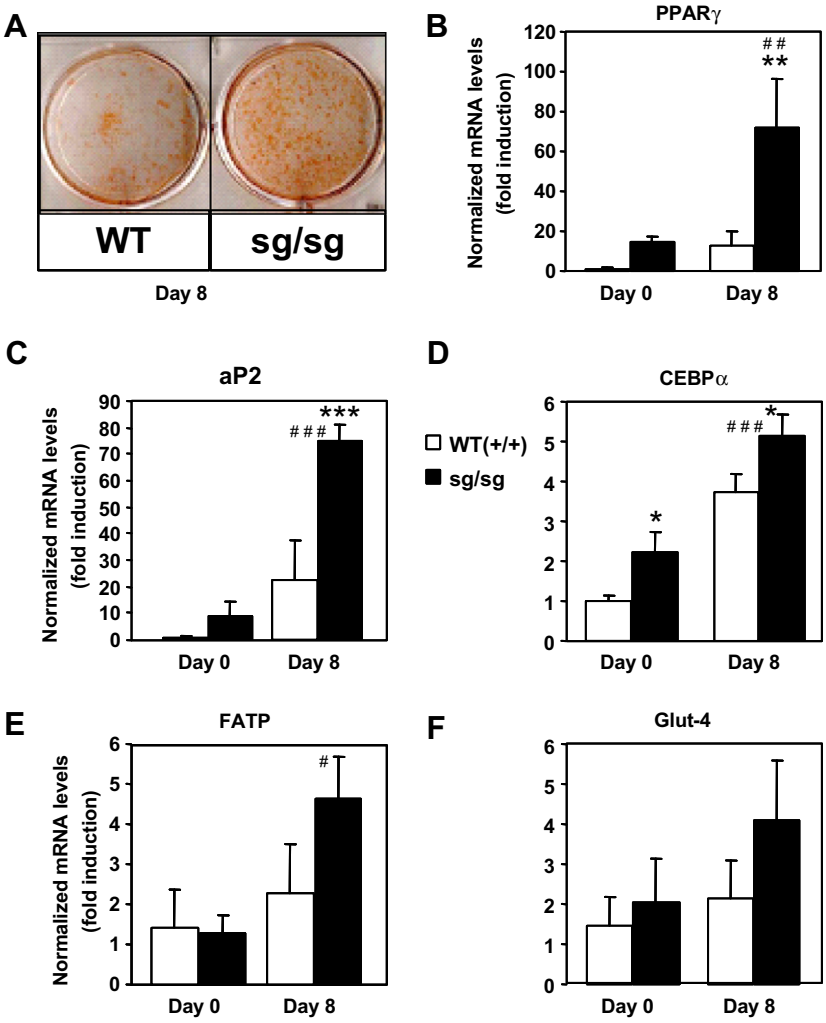

Fig. 4. RORα-deficiency improves adipocyte differentiation and function. Oil-red-O staining (A), mRNA levels of genes involved in adipogenesis (B–D) and FA (E) and glucose uptake (F) in differentiating MEFs isolated from staggerer (sg/sg) ($n = 4$) and wild-type (WT, +/+) ($n = 4$) mouse embryos. Results are from three independent wells (per embryo) and expressed as fold induction of wild-type cells at day 0, set as 1. *Difference between wt and sg/sg MEFs; #difference between day 0 and day 8.

more than two groups were by ANOVA followed by pair-wise Tukey post hoc analysis. $^{\#,*}P < 0.05$; $^{\#\#,**}P < 0.01$; $^{\#\#\#,***}P < 0.001$.

3. Results

3.1. RORα1 and RORα4 are expressed in adipose tissue and differentiating adipocytes

Expression of the four RORα isoforms (RORα1–4) was analyzed in both human and mouse visceral and subcutaneous adipose tissue by quantitative PCR. RORα1 and RORα4 were abundantly expressed in these tissues, whereas RORα2 or RORα3 were not detected (not shown). RORα4 mRNA levels were ~10-fold higher than RORα1 mRNA levels in both intra-abdominal and subcutaneous adipose tissues (Fig. 1A and B). In the 3T3-L1 adipocyte differentiation model, both RORα1 and RORα4 mRNA levels increased during differentiation, but RORα4 induction was much larger (Fig. 1C). Because the PCR efficiency may differ between the two pairs of primers, the absolute Ct values are given in Table S1.

3.2. RORα4 over-expression impairs differentiation of 3T3-L1 pre-adipocytes

To assess a potential role of RORα4 in adipocyte differentiation, 3T3-L1 pre-adipocytes stably over-expressing the full-length human RORα4 (MFG-RORα4) or control cells (MFG-Neo) were generated. RORα4 expression was significantly higher in MFG-RORα4 compared to MFG-Neo cells, both at the mRNA and protein level (Fig. 2A and B). Oil-red-O staining visualization at different stages during differentiation showed, as expected, time-dependent enhancement of lipid accumulation in MFG-Neo cells (Fig. 2C). Strikingly, a marked reduction in lipid accumulation was observed in RORα4-overexpressing adipocytes (Fig. 2C). This impairment was associated with reduced expression of several genes involved in adipocyte differentiation, such as PPARγ, CEBPα, aP2 and ADD1/SREBP1 (Fig. 2D–G).

3.3. RORα4 suppresses glucose and free fatty acid uptake in adipocytes

The function of adipocytes in the control of lipid and glucose homeostasis is well-established. Therefore, the influence of RORα4 on free acid fatty (FFA) and glucose uptake was tested in differentiated 3T3-L1 cells. Compared to control cells, RORα4 over-expressing adipocytes showed an impaired ability in insulin-stimulated uptake of FFAs (Fig. 3A). Consistently, gene expression of CD36 and FATP, two FA transporters induced during adipocyte differentiation, was strongly reduced by RORα4 over-expression (Fig. 3C and D). Similarly, RORα4 over-expression negatively regulated expression of genes involved in de novo fatty acid synthesis and glycerogenesis such as FAS, ACC and PEPCK (Fig. 3F–H). In

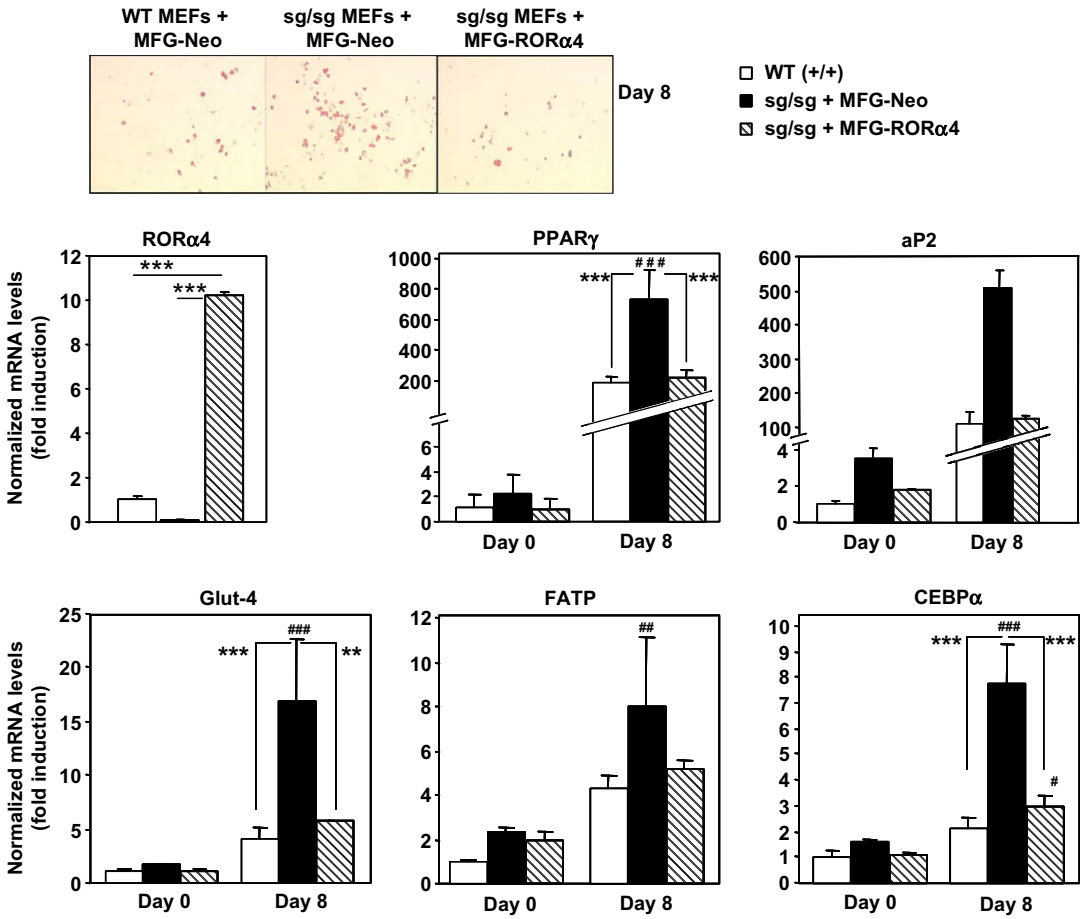

Fig. 5. RORα4 restoration in RORα-deficient MEFs abolishes the permissive effects of RORα-deficiency. Oil-red-O staining and mRNA levels of RORα4 and genes involved in adipogenesis, FA and glucose uptake in differentiating MEFs isolated from wild-type ($n = 2$) and sg/sg ($n = 2$) mouse embryos and over-expressing RORα4 (sg/sg-MFG-RORα4) or a control (sg/sg-MFG-Neo) construct. Results are from three independent wells (per embryo) and expressed as fold induction of wild-type cells at day 0, set as 1. *Difference between wt, sg/sg-MFG-Neo and sg/sg-MFG-RORα4 MEFs; #significant difference between day 0 and day 8.

addition, RORα4 over-expression significantly reduced insulin-stimulated glucose uptake in differentiated adipocytes (Fig. 3B), an effect which was associated with a suppression of expression of the glucose transporter Glut-4 (Fig. 3E). Overall, these observations indicate that RORα4-induced impairment of adipocyte differentiation is accompanied by altered FA and glucose metabolism.

3.4. RORα-deficiency improves adipocyte differentiation and function

Next, we assessed whether RORα-deficiency modulates adipocyte differentiation using MEFs from *staggerer* (*sg/sg*) mice which carry a non-functional mutated RORα gene. MEFs from *staggerer* mice display a more pronounced lipid accumulation indicative of increased differentiation into adipocytes compared to MEFs from their wild-type littermates (Fig. 4A). This increased ability of *sg/sg* MEFs to accumulate lipids was associated with an increased expression of several markers of adipocyte differentiation such as PPARγ, aP2 and CEBPα (Fig. 4B–D) or genes involved in fatty acid and glucose uptake such as FATP and Glut-4 (Fig. 4E and F). RORα4 restoration in MEFs from sg/sg mice blocked the permissive effects of RORα-deficiency on lipid accumulation and adipocyte differentiation, as well as expression of the PPARγ, CEBPα, aP2, FATP and Glut-4 genes (Fig. 5).

4. Discussion

Previous studies have identified a role for RORα in cerebellum development, immune function and circadian rhythmicity [11–13,22]. Recent reports have also outlined a function for RORα in cholesterol and lipid metabolism [14,15,23]. In the present study we show that the RORα1 and RORα4 genes are expressed in adipose tissue and that RORα increases upon differentiation of pre-adipocytes into adipocytes, identifying RORα4 as the principal isoform in adipose tissue. Moreover, RORα4 over-expression in 3T3-L1 cells inhibits adipocyte differentiation, impairs fatty acid and glucose uptake and reduces expression of genes known to be involved in both adipocyte differentiation (including PPARγ, CEBPα and aP2) and function (such as FAS, PEPCK, and the fatty acid and glucose transporters FATP, CD36 and Glut-4).

Staggerer mice carry a naturally occurring mutation in the ligand-binding domain of RORα, which results in the expression of a non-functional RORα protein. These mice suffer from cerebellar ataxia and have reduced locomotor activity [24]. These mice additionally exhibit altered cholesterol and triglyceride plasma levels and increased susceptibility to atherosclerosis and angiogenesis [15,25]. Interestingly, Muscat and co-workers have recently reported that *staggerer* mice display reduced fat depots and are resistant to diet-induced obesity [26]. The authors attributed this defect to alteration in lipid metabolism as a result of down-regulated expression of SREBP1c, FAS, ABCA1 and ABCG5 in liver, and increased expression of genes involved in oxidative metabolism in liver and adipose tissue. However, altered locomotor and rearing activity of these mice coupled to the fact that they need special assistance for food and drink intake may also have impacted this phenotype. We show that MEFs isolated from *staggerer* mice differentiate more pronouncedly into mature adipocytes compared to

MEFs isolated from wild-type littermates. This effect is specific to RORα since the phenotype of sg/sg MEFs is reversed by RORα restoration. Thus, RORα4 is a potent negative regulator of adipocyte differentiation and may as such play a role in body weight and energy homeostasis.

Although our experiments did not address the molecular mechanism(s) involved in the RORα-mediated inhibition of adipogenesis, several hypotheses can be put forward. Inhibition of adipocyte differentiation may occur principally through inhibition of positive regulators such as PPARγ or CEBPα, or through the induction of inhibitory factors like GATA, KLF2, CHOP or Wnt signaling [3]. Alternatively, RORα may regulate other factors known to inhibit adipocyte differentiation, for instance, through induction of $p21^{CYP1/Waf1}$ leading to growth arrest. Along this line, Rev-erbα acts as a p21 repressor in hepatic cells [27], and RORγ induces p21 in liver. Thus, RORα might act, at least in part, by up-regulating p21 transcription in adipose cells. Another possible explanation may lie in the recent observation that Rev-erbα represses PPARγ2 gene expression during adipocyte differentiation [6]. The fact that RORα induces Rev-erbα gene transcription ([28] and this report, not shown) may constitute an additional potential mechanism for adipogenesis inhibition by RORα.

Although future studies are necessary to further delineate RORα-regulated pathways in adipose cells, our findings clearly identify RORα4 as novel negative modulator of adipocyte differentiation and function.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2009.05.019.

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